Gene, 167 (1995) 17–24

GENE 09398

Characterization of two members (*ACS1* and *ACS3*) of the 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*

(Ethylene biosynthesis; multigene family; pseudogene; gene duplication; gene evolution; amino acid deletions)

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Received by J. Messing: 19 May 1995; Accepted 21 August 1995; Received at publishers: 28 September 1995

ABSTRACT

The nucleotide sequences of two highly homologous 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS; EC 4.4.1.14)-encoding genes, *ACS1* and *ACS3*, of *Arabidopsis thaliana* (*At*) have been determined. The sequence analysis shows that *ACS3* is a pseudogene representing a truncated version of *ACS1*. The missing region of *ACS3* corresponding to the fourth exon of *ACS1* has been shown by Southern analysis to be absent in the *At* genome. The chromosomal locations of the five members of the *At ACS* multigene family have been determined. The results show that each family member resides on a different chromosome. This observation suggests that the *ACS3* pseudogene originated by a partial inter-chromosomal gene duplication. The ACS1 polypeptide contains all the conserved and characteristic domains found in the ACC synthase isoenzymes from various plant species, but is unable to express ACS activity in *Escherichia coli* and yeast. The predicted amino-acid sequence of ACS1 is missing the highly conserved tripeptide, Thr-Asn-Pro (TNP), between Ile^{204} and Ser^{205}. Introduction of TNP into ACS1 restores the ACS activity, whereas its removal from the enzymatically active ACS2 results in a loss of activity. The results suggest that TNP is crucial for expression of ACS activity in *E. coli*.

INTRODUCTION

Ethylene, one of the simplest organic molecules with biological activity, is involved in controlling many aspects of plant growth, development and senescence (Yang and Hoffman, 1984; Abeles et al., 1992). The rate-limiting step in the biosynthesis of ethylene is the conversion of *S*-adenosyl-L-methionine (SAM) to

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Abbreviations: aa, amino acid(s); ACC, 1-aminocyclopropane-1-carboxylic acid; *ACS*, gene encoding ACS; ACS, 1-aminocyclopropane-1-carboxylate synthase(s); AmT, aminotransferase(s); *At*, *Arabidopsis thaliana*; bp, base pair(s); cDNA, DNA complementary to RNA; Chx, cycloheximide; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); p, plasmid; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; re-, recombinant; RFLP, restriction-fragment length polymorphism; SDS, sodium dodecyl sulfate; TNP, Thr-Asn-Pro tripeptide between Ile^{204} and Ser^{205} absent in ACS1; *tsp*, transcription start point(s); *UTR*, untranslated region; [ ], denotes plasmid-carrier state.

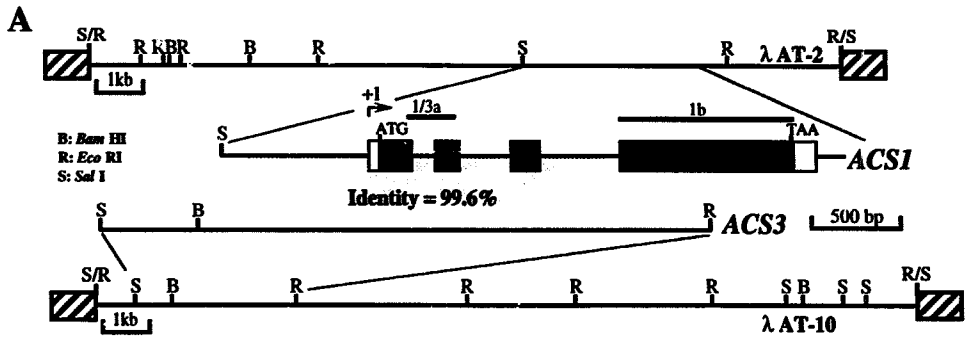
1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, catalyzed by the enzyme ACC synthase (SAM-methylthioadenosine-lyase, EC 4.4.1.14, ACS). ACC synthase activity is highly regulated during plant development and is induced in response to a wide variety of environmental stimuli including wounding, anaerobiosis, viral infection, and elicitor treatment. Ethylene itself, as well as other plant hormones such as auxin, are also known to enhance ethylene production and ACC synthase activity (Yang and Hoffman, 1984; Abeles et al., 1992). Recent studies using cloned *ACS* genes from several plants indicate that the induction of the enzyme activity is due to an increase in the accumulation of its mRNA (Kende, 1993; Zarembinski and Theologis, 1994). Using *At*, we have sought to develop experimental strategies for genetically dissecting the components of signal transduction pathways responsible for the activation of the various *ACS* genes (Estelle and Somerville, 1986). As a first step toward this goal, we have previously isolated and characterized the multigene family encoding ACC synthase in *At*. We have also shown that the gene family consists of at least five divergent members which are differentially regulated during development and in response to inducers such as auxin, Li^+ and cycloheximide (Liang et al., 1992; Abel et al., 1995). Here we describe the structural characteristics of two highly homologous *ACS* genes, *ACS1* and *ACS3* (Liang et al., 1992), and the chromosomal organization of the five isolated *ACS* genes in *At*.

RESULTS AND DISCUSSION

(a) Structural characterization of *ACS1* and *ACS3*

We have previously isolated five genomic sequences from *At* coding for ACS (Liang et al., 1992). Restriction analysis of genomic clones and sequence comparison of DNA fragments generated by PCR have revealed that two of the *At ACS* genes, *ACS1* and *ACS3*, are highly homologous in their 5′ region, and diverge considerably in the 3′ and flanking regions (Liang et al., 1992). Further structural characterization of the *ACS1* and *ACS3* genes have been carried out using the previously isolated genomic clones λAT-2 (*ACS1*) and λAT-10 (*ACS3*) (Fig. 1A). The 3340-nt sequence of *ACS1* is shown in Fig. 1B. The intron/exon junctions have been established by reference to other cloned *ACS* genes and confirmed by comparison with the sequence of an *ACS1* cDNA, paACS1, which has been generated by PCR using synthetic primers and a reverse-transcribed cDNA template. The coding 1467-nt region is interrupted by three introns at positions conserved in the majority of the *ACS* genes (Fig. 1B;

Fig. 1. Organization of the *ACS1* and *ACS3* genes. (**A**) partial restriction maps. The 4.5-kb *Sal*I-*Eco*RI fragment of λAT-2 and the 3.2-kb *Sal*I-*Eco*RI fragment of λAT-10 were subcloned into pUC18, respectively. Dideoxy sequencing of double-stranded DNA of both subclones was performed with universal and synthetic primers using [^{35}S]dATP (Sanger et al., 1977) and the modified T7 DNA polymerase, Sequenase, according to the manufacturer's instructions (US Biochemical). Both strands of the indicated regions of λAT-2 (*ACS1*) and λAT-10 (*ACS3*) were sequenced. The coding regions of *ACS1* are filled blocks and the 5′-and 3′-UTR are open blocks. The +1 and arrow indicate the start and direction of transcription in *ACS1*. The *ACS1* and *ACS3* genes are aligned according to the nearly identical region which is highlighted with the shaded block. The lines above the *ACS1* gene represent the two PCR-generated probes, 1/3a and 1b, used in the genomic Southern analysis shown in Fig. 2. (**B**) The nt sequence of the *ACS1* gene, including introns and 5′- and 3′-*UTR*, in comparison with the partial sequence of the *ACS3* gene. The highly homologous sequences found in both genes are shaded according to the gene structures shown in A. The *ACS3* sequence contains the entire homologous region and partial flanking sequences, only the nt different from the corresponding *ACS1* sequence are shown. The *ACS1* mRNA sequence was defined by comparing the sequence of RT-PCR fragments with the genomic sequence. RT-PCR fragments were generated using poly(A)$^+$RNA from Chx-induced *At* seedlings, as a template, and the following amplimers: geneA5′ (5′-CATGCCTGCAGGATGTCTCAGGGTGCATG), geneA3′ (5′-GTGACGAATTCTTAAGCTCGAAGCAATGG). The paACS1 cDNA was generated using these PCR amplified fragments, and its sequence is identical to those of the *ACS1* exons except for a single $A^{369}\rightarrow G$ change (resulting in no change of the encoded aa). The nt +1 corresponds to the *tsp* of *ACS1*, which has been determined by 5′-RACE. The experiment was performed essentially as described by Bahring et al. (1994) using *ACS1*-specific primers, YF-2 (from +469 to +452) and YF-4 (from +239 to +211) both underlined in Fig. 1B, and cDNA generated from poly(A)$^+$RNA isolated from Chx-induced *At* seedlings as template. The nts upstream from the +1 position are negatively numbered. The polyadenylation site of *ACS1* was determined by comparison of genomic DNA sequence with that of the cDNA fragment generated by inverse PCR (Huang et al., 1990) using double-stranded circular cDNA as template (Liang et al., 1992) and primers YF-2 and ACS1s1 (from nt +2016 to +2022, indicated by underline) as amplimers. The mRNA encoded by the *ACS1* gene is shown in capital letters. The remainder of the sequence is shown in small letters. The derived aa sequence is presented in the one-letter code below the nt sequence and is numbered separately. The 11 boxed residues are the invariant aa conserved among various subgroup-I aminotransferases (Rottmann et al., 1991; Mehta et al., 1993). The underlined dodecapeptide beginning at Ser^{270} is part of the active center of ACC synthase (Yip et al., 1990). The TATA box and putative polyadenylation signals (AATATAA) are boxed. The translation initiation codon (ATG) is double underlined. The sequences reported in this paper have been deposited in the EMBL/GenBank database with the following accession Nos.: *ACS1*, U26542; *paACS1*, U26543; *ACS3*, U26544. (**C**) The aa alignment of the region surrounding the TNP tripeptide from various *At* ACC synthases. The sequences are: At, ACS1 (this paper), ACS2 (Liang et al., 1992), ACS4 (Abel et al., 1995) and ACS5 (Liang et al., unpublished). The corresponding region of the chicken mitochondrial aspartate AmT (AspATmc; Graf-Hausner et al., 1983) and the predicted secondary structures of the two classes of enzymes are shown at the bottom. TNP is indicated by arrowheads. Solid circles designate those aa that are invariant in subgroup-I AmT. The aa of ACS are numbered based on the ACS1 sequence. The secondary structure of ACC synthase was obtained by e-mailing the protein sequences to 'predictprotein@embl-heidelberg.de' (Rost and Sander, 1993). The symbols used for secondary structure are: H, helix; E, extended (strand); and blank, rest (loop).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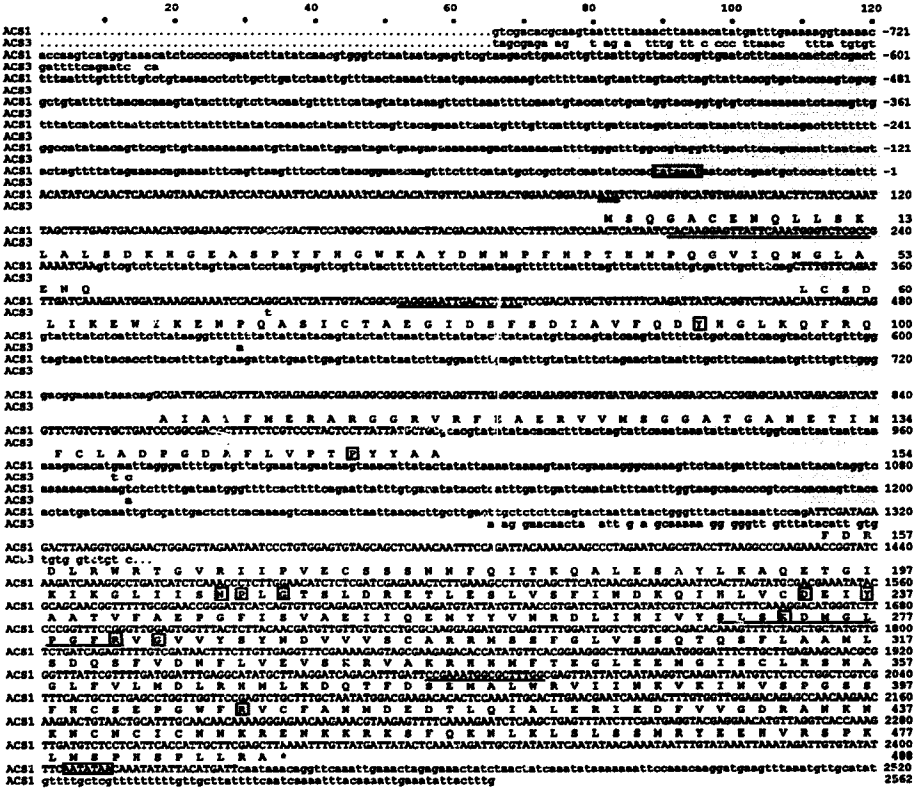

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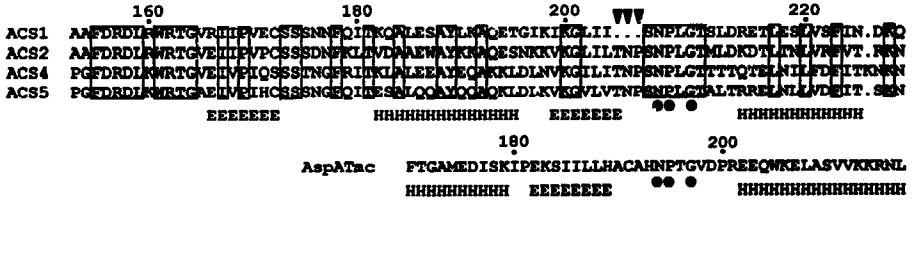
ACS1
ACS2
ACS4
ACS5
AspATac
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Lincoln et al., 1993). The *tsp* has been determined by 5′ RACE with poly(A)$^+$RNA from cycloheximide-treated *At* seedlings (Liang et al., 1992). The polyadenylation site has been established by comparing the 3′-genomic sequence of *ACS1* with that of a cDNA fragment generated by inverse PCR (Fig. 1B; Huang et al., 1990). The 5′- and 3′-UTR of the *ACS1* gene are thus defined as 80 nt and 112 nt in size, respectively. The *ACS1* gene encodes a protein of 488 aa (55.0 kDa, p*I* 7.2) that has all the characteristic features of ACC synthase including the well-conserved dodecapeptide in the reaction center (Yip et al., 1990) and the invariant 11 aa that are conserved among various subgroup-I aminotransferases (Rottmann et al., 1991; Mehta et al., 1993). The homology between *ACS1* and other isolated *ACS* genes ranges from 49% to 66% in identity. The sequence of 3158 nt of *ACS3*, part of which is shown in Fig. 1B, has also been determined. Comparative analysis between *ACS1* and *ACS3* shows that a 1967 nt sequence of *ACS3* is almost identical (99.6% in identity) to a region of *ACS1* containing 726 nt of promoter sequence, entire exons 1, 2 and 3, introns 1 and 2, and part of intron 3 (Fig. 1A,B).

(b) The defective structure of *ACS3*

The nt sequence of *ACS3* diverges extensively from that of *ACS1* outside the 1967-nt region (Fig. 1B). The 3′-boundary of the homology between the two ends just before the junction of intron 3 and exon 4 of the *ACS1* gene (Fig. 1B). Sequence analysis of the downstream region has not revealed further homology. To examine whether the 3′ region of ACS3 is present in the *At* genome, two DNA probes have been generated by PCR. Probe 1/3a corresponds to a region covering the *ACS1* intron 1 and part of exon 2, whereas probe 1b corresponds to the fourth exon which is missing in the *ACS3* sequence (Fig. 1A). Genomic Southern analysis using the two probes demonstrates that while 1/3a hybridizes to two genomic DNA fragments corresponding to *ACS1* and *ACS3* respectively (Fig. 2A), the 1b probe detects only the fragment containing *ACS1* (Fig. 2B). Thus, the *ACS1* exon 4 represents the only copy of the sequence in the *At* genome. This has lead to the conclusion that *ACS3* is a pseudogene representing a truncated version of *ACS1*.

(c) Expression of *ACS1* in *E. coli*

Four *At ACS* cDNAs corresponding to *ACS1*, *ACS2*, *ACS4*, and *ACS5* were cloned into pPO9, a pET derived non-fusion type expression vector (Rottmann et al., 1991; Studier et al., 1990) and expressed initially in *E. coli* BL21(DE3) (Studier et al., 1990). However, we were unable to obtain transformants with pPO9-ACS5 using BL21(DE3) as a host (data not shown). Probably

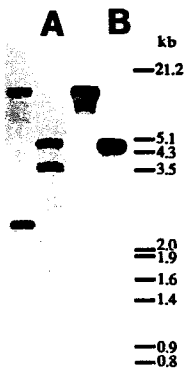

Fig. 2. Southern analysis. *At* genomic DNA (5 µg) was digested with *Bam*HI + *Sal*I (lane 1) and *Eco*RI + *Sal*I (lane 2), electrophoresed in 0.8% (w/v) agarose gel, transferred to Nytran membrane (Schleicher & Schuell), and hybridized with ^{32}P-labeled 1/3a (A) and 1b (B) probes (Fig. 1A). The size markers shown on the right are λ DNA digested with *Eco*RI + *Hin*dIII.

overexpression of *ACS5* is toxic to *Escherichia coli*. We were able, however, to obtain ACS5 transformants in BL21(DE3)[pLysS] or BL21(DE3)[pLysE], in which protein production is reduced by T7 lysozyme (Studier et al., 1990). Surprisingly, all ACC synthases except ACS1 express ACC synthase activity after 6 h of incubation in the presence of 1 mM IPTG (Fig. 3, panel IA). Furthermore, immunoblotting analysis shows that the *E. coli* strain expressing the *ACS1* cDNA does not accumulate ACS1 protein, whereas the ACS2, ACS4, and ACS5 proteins are readily detectable (Fig. 3, panel III, compare lane 2 with lanes 3, 4, and 5, respectively). Similar results were obtained with a *lacZ-ACS1* fusion using pUC19 or with *s10-ACS1* fusion using the pET system (Studier et al., 1990) expressed in various *E. coli* strains (data not shown).

(d) Inactivity of ACS1

The aa alignment of the four *At* ACC synthase isoenzymes ACS1 (55.0 kDa, p*I* 7.2), ACS2 (55.5 kDa, p*I* 7.3), ACS4 (53.8 kDa, p*I* 8.4), and ACS5 (53.3 kDa, p*I* 7.5) shows that the ACS1 is missing the tripeptide TNP between Ile^{204} and Ser^{205} (see Fig. 1C). The absence of TNP is not due to a cloning artifact because both the genomic and the cDNA sequences contain the deletion.

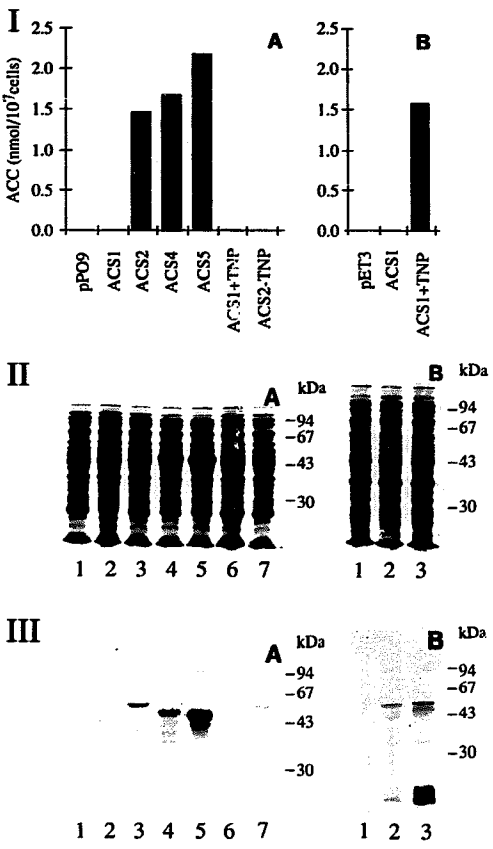

Fig. 3. Synthesis of the *At* ACC synthase isoenzymes in *E. coli*. **Panel I**: Accumulation of ACC in (**A**) *E. coli* BL21(DE3)[pLysE] expressing the isoenzymes ACS1, ACS2, ACS4, ACS5, ACS1+TNP, and ACS2-TNP with the pPO9 expression vector; and (**B**) *E. coli* BL21(DE3) expressing ACS1 and ACS1+TNP with the pET3c expression vector. **Panel II A and B**. Coomasie blue stained gels with *E. coli* protein extracts from induced recombinant clones shown in panel IA and B, respectively. **Panel III A and B**: Immunoblotting analysis of replica gels as shown in panel IIA and B, respectively, with antibody to the tomato isoenzyme LE-ACS2. **Methods:** The four *At* cDNA fragments paACS1, paACS2, paACS4, and paACS5 were cloned as *NdeI-BamHI* fragments into pPO9 vector. The *NdeI* site is located at the ATG start codon of each cDNA. The recombinant plasmids were introduced into *E. coli* BL21(DE3)[pLysE]. The pPO9-ACS1+TNP was generated by PCR using ACS1+TNP(5'-CAAAGGCCTGATCATCACCAACCCATCAAACCCTCTTGG) and the amplimer, ACS1aSB (5'-TTGCAGAGCTCGGATCCTTAAGCTCGAAGCAATG). The amplified fragment was digested with *StuI*+*BamHI* and ligated with the *NdeI-StuI* fragment of *ACS1*, then the *NdeI-BamHI* fragment was subcloned into pPO9. For the construction of pPO9-ACS2-TNP a *StuI* site was introduced into the cDNA by PCR. Two fragments were generated by PCR: (*i*) An *NdeI-StuI* fragment using the amplimers ACS2sBN(5'-GGATGAGATCTTCCATATGGGTCTTCCGGGAAAAAATAAAGG) and ACS2aSt(5'-ATCAGGCCTTTGACTTTTTTATTGGAC) and (*ii*) A *StuI-ClaI* fragment with the amplimers ACS2-TNP(5'-CAAAGGCCTGATTTTGTCAAATCCACTCGGTACAATG) and ACS2aSB(5'-TTGCAGAGCTCAGATCTTCATGCTCGGAGAAGAGGTG). The amplified fragments were ligated and subcloned to *NdeI*+*ClaI*-cleaved pPO9-ACS2. The pET3c expression constructs of ACS1 and ACS1+TNP were synthesized by PCR and introduced as *BamHI* fragments into the vector. The PCR-made ACS1+TNP has a G instead of an A^{1005} and the ACS2-TNP has a $C^{383} \rightarrow$ A transversion. Both changes do not affect the encoded aa. The ACC accumulation was determined as described by Rottmann et al. (1991) except that one A_{600} unit is assumed to correspond to 6×10^8 cells/ml. For 0.1% SDS-10% PAGE analysis, total protein extracted from 5×10^7 cells was loaded per lane. Immunoblotting was carried out using the tomato LE-ACS2 antibody (Ab) (Rottmann et al., 1991) purified through a column containing total *E. coli* proteins (Sambrook et al., 1989). An alkaline phosphatase-conjugated goat anti-rabbit IgG was used as a secondary Ab.

The tripeptide TNP is highly conserved among all the cloned ACS synthases from various plant species. Expression of hybrid proteins between ACS1 (inactive) and ACS2 (active, reveals that the hybrids ACS1(Met^1-Lys^{200})/ACS2(Gly^{202}-Ala^{496}) and ACS2(Met^1-Asp^{217})/ACS1(Arg^{214}-Ala^{488}) are enzymatically active in *E. coli*, whereas the hybrids ACS1(Met^1-Asp^{213})/ACS2(Lys^{218}-Ala^{496}) and ACS2(Met^1-Lys^{201})/ACS1(Gly^{201}-Ala^{488}) are inactive (data not shown). These results indicate that the region between Gly^{201} and Asp^{213} of ACS1 is responsible for its inactivity. To directly demonstrate that the inactivity of ACS1 is due to the deletion of the tripeptide TNP, we engineered an *ACS1+TNP* isoenzyme by inserting the tripeptide between Ile^{204} and Ser^{205}. We also constructed an ACS2 isoenzyme (active), *ACS2-TNP*, without the conserved tripeptide. The modified isoenzymes were expressed in *E. coli* BL21(DE3)[pLysE] with the pPO9 expression vector, and the results are shown in Fig. 3IA. Deletion of TNP from ACS2 abolishes its activity, whereas addition of TNP into ACS1 results in very low activity. Immunoblotting experiments show that the enzymatically active ACS2 and inactive ACS2-TNP accumulate in *E. coli*, whereas both ACS1 and ACS1+TNP are undetectable. These results raised the possibility that the ACS1 inactivity may be associated with extreme instability of this isoenzyme in *E. coli*. However, we subsequently observed that expression of ACS1+TNP in a different *E. coli* strain BL21(DE3) with a different expression vector (pET3c) resulted in recovering enzymatic activity compared to ACS1 (Fig. 3IB). Furthermore, immunoblotting experiments show that the inactivity of ACS1 is not due to its inability to accumulate in *E. coli* because the polypeptide can be immunodetected (Fig. 3IIIB, lane 2). The gain of activity of ACS1+TNP coincides with an enhanced accumulation of the polypeptide in *E. coli* (Fig. 3IIIB). Furthermore, large accumulation of degraded ACS1+TNP is also observed (Fig. 3IIIB). The data suggest that the tripeptide TNP

may be a stability determinant because its addition to ACS1 enhances the amount of the isoenzyme, whereas its deletion from ACS2 considerably lowers its expression (compare lane 2 with 3 in Fig. 3IIIB and lane 3 with 7 in Fig. 3IIIA). Addition of the TNP also reverses the inactive ACS1 into an active isoenzyme in *Saccharomyces cerevisiae* (Table I).

The sequence surrounding the TNP tripeptide is highly conserved in various ACC synthases (data not shown) and also in subgroup-I aminotransferases (AmT), which are close relatives of ACS (Mehta and Christen, 1994; Rottmann et al., 1991). Although TNP is not conserved in the AmT (Fig. 1C), secondary structure analysis reveals that ACS and AmT have very similar secondary structures around the TNP tripeptide (Fig. 1C). According to tertiary structure of the chicken mitochondrial aspartate AmT, a member of the subgroup-I AmT, the loop between Asn^{194} (Asn^{206} in ACS1) and Gly^{197} (Gly^{209} in ACS1) participates in the active site where Asn^{194} interacts with the 3'-hydroxyl group of the pyridine moeity of the PLP co-factor (McPhalen et al., 1992). The possibility exists that, because of the similar predicted secondary structure between ACS and AmT, a similar loop is present in ACS. Since the TNP is located at the end of this putative loop, its absence may be responsible for the inability of Asn^{206} to interact with the PLP cofactor and thus causes its inactivity. Furthermore, since *ACS1* is transcriptionary active in *At* (Liang et al., 1992), the question arises whether the ACS1 polypeptide is also inactive in the intact plant and what is its precise role during growth and development. The possibility exists that the ACS1 polypeptide may be posttranslationally modified and is indeed active in the intact plant. It may also act as a dominant negative regulator of other ACS by forming heterodimers (Herskowitz, 1987). The purification and biochemical characterization of the native ACS1 protein from intact *At* plants will address these various possibilities.

TABLE I

Expression of *ACS1* and *ACS1+TNP* in *Saccharomyces cerevisiae*[a]

Construct (plasmid)	ACC accumulation (nmol/10^7 cells)/24 h
pBM258	0.02±0.00
pBM258-ACS1	0.04±0.02
pBM258-ACS1+TNP	0.63±0.08

[a] The coding regions of *ACS1* and *ACS1+TNP* were subcloned into the *Bam*HI site of the yeast expression vector pBM258 (Johnston and Davis, 1984) and introduced into yeast strain YM2061. The yeast cells were grown in YP media at 30°C for 24 h containing 2% galactose. The ACC released into the medium was assayed according to Lizada and Yang (1979).

(e) Chromosomal assignment of the *At ACS* genes

Various pseudogene sequences generally fall into two categories: (*1*) those that retain the intervening sequences found in their functional counterparts and (*2*) processed pseudogenes (Vanin, 1985). Apparently *ACS3* belongs to the former. Plant pseudogenes of this kind have been found, for example, in the leghemoglobin gene family of soybean (Brisson and Verma, 1982), H4 histone genes of tomato (Kosciessa and Doenecke, 1990), and a small subunit of ribulose bisphosphate carboxylase genes of tobacco (O'Neal et al., 1987). This type of pseudogenes are often present in gene clusters and believed to be resulted from gene duplication (Proudfoot, 1980). *ACS3* is the first pseudogene found in ACC synthase gene family. However, beside the finding of a pair of highly homologous and closely linked *ACS* genes in zucchini (Huang et al., 1991), tomato (Rottmann et al., 1991), and potato (Destéfano-Beltrán et al., 1995), there is no further evidence indicating clustering of *ACS* genes in the *At* genome. By RFLP analysis using a population of 117 re-inbred lines (Reiter et al., 1992a,b), we have mapped the five isolated *ACS* genes on the *At* chromosomes. The results are shown in Fig. 4. It appears that each individual *ACS* gene occupies a different *At* chromosome with *ACS1* on chromosome 3, *ACS2* on chromosome 1, *ACS3* on chromosome 4, *ACS4* on chromosome 2 and *ACS5* on chromosome 5, respectively (Fig. 4). Interestingly, *ACS1* and *ACS3* are located on two different chromosomes, suggesting that the gene duplication is also coupled with an inter-chromosomal translocation.

(f) Evolution of the *At ACS1* and *ACS3* genes

The nearly identical nt sequence between *ACS1* and *ACS3* is observed throughout the 5'-flanking, 5'-untranslated, intervening and coding regions of the *ACS1* gene (Fig. 1B), indicating that the duplication of *ACS3* from *ACS1* is a very recent evolutionary event. However, it is still not clear whether the gene duplication and the subsequent inter-chromosomal translocation happened as a single event or as two independent events. It should be pointed out that there are no repeats of any kind found in the nt sequence flanking the nearly identical regions. This observation raises the question whether the two *At* genes once existed as a counterpart of the closely linked gene pair found in zucchini (i.e., *CP-ACS1A/1B*; Huang et al., 1991), tomato (i.e., *LE-ACS1A/1B*; Rottmann et al., 1991) and potato (i.e., *ST-ACS1/B*; Destéfano-Beltrán et al., 1995). This seems highly unlikely since the two clustered genes involved in the last three cases diverge significantly in the flanking, untranslated and intervening sequences, implying that the related gene duplication happened much earlier in evolution. In addition, the remarkable homology found in the

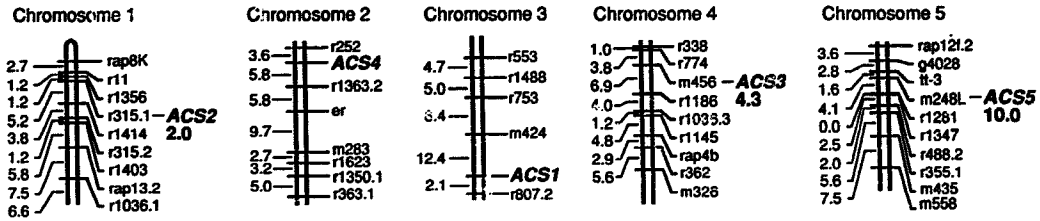

Fig. 4. Chromosomal assignment of the *At ACS* genes. Marker loci along the chromosome bars were ordered with a logarithm of odds (LOD) score difference >3.0, markers to the right of each bar could not be ordered with equal confidence (LOD score difference <3.0). Distances between markers in cM (centiMorgans) are shown on the left of each chromosome bar. For markers ordered with a LOD score less than 3.0, approximate distance from the corresponding LOD 3.0 marker is shown below. DNA from *ACS* λ clones (Liang et al., 1992) were mapped by RFLP analysis in a population of 117 inbred re-lines as described by Reiter et al. (1992a,b).

nt sequence of the coding regions of the two closely linked genes is indicative of concerted evolution (Dover et al., 1993). Our failure to find the 3′ portion of the more recently created *ACS3* gene in the *At* genome argues that the gene may have never existed as a intact and functional *ACS* gene. Thus, unlike the duplicated *ACS* genes of zucchini, tomato, and potato, the *At ACS3* resulted probably from a very recent partial inter-chromosomal gene duplication and may have never had an opportunity to co-evolve with its ancestral *ACS1* gene. It is simply waiting for its eventual obliteration as one of the 'relics of evolution'.

(g) Conclusions

(*1*) The five family members of the *ACS* gene family of *At* reside on different chromosomes.

(*2*) The *ACS3* gene, a homolog of *ACS1*, is a pseudogene originated by a partial interchromosomal gene duplication.

(3) The *ACS1* is missing the highly conserved tripeptide TNP between Ile^{204} and Ser^{205} resulting in inactivity when expressed in *E. coli* and yeast.

ACKNOWLEDGMENTS

This work was supported by grants to A.T. from the National Science Foundation (MCB-9316475) and the U.S. Department of Agriculture (5335-21430-00300D). Y.O. acknowledges the support of a JSPS Fellowship for Japanese Junior Scientists. We thank Ron Wells for editing the manuscript.

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